Pre-Submission Seminar on:

"*In vitro* propagation and phytochemical screening of three important ethno-medico-botanicals used by the ethnic communities of Purulia district, West Bengal, India"

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Title of the study:

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1. Introduction:

Since time immemorial, plants and natural products have been used in various indigenous systems of medicine throughout the world to reduce human mortality and morbidity. In many countries such alternative treatment strategies complement modern healthcare systems (Yigezu et al., 2014). Traditional systems of medicine have inspired the search of lead compounds with pharmacological potentials in the treatment of many ailments (Zofou et al., 2013; Ahmed and KabidulAzam, 2014). Ethnopharmacology may serve as the first evidence of therapeutic efficacy of plants which might be utilized in drug discovery programs to compete with the conventional synthetic medicine. Three-fourth of the world's population is dependent on indigenous medicine (Lawson 1979). World Health Organization (WHO) has also recognized the role of traditional medicine in the primary health care system (Akerele 1984; Anonymous 1991).

A number of tribal people with diverse cultural backgrounds reside in India and practice their own system of traditional medicine for primary healthcare. Reportedly 80% of the Indians use non-allopathic (Ayurveda, Siddha, Unani and Homeopathy) medicines for their healthcare and herbs constitute a major ingredient of these alternative systems of medicine (Gogtay et al. 2002). Synergistic botanical preparations used by the traditional healers (THs) may be considered as one of the prime strategies in drug discovery (Patwardhan and Mashelkar 2009).In India, non-allopathic treatment strategies [also known as complementary and alternative medicine (CAM)] constitute almost 80% of the population's medical need. Non-allopathic medications in India include Ayurveda, Siddha, Unani and Homoeopathy. Ayurveda is considered to be the oldest (since 5000 B.C.) and the most popular healthcare option in rural India (Gogtay et al., 2002). Besides Ayurveda, other aboriginal and traditional use of medicinal plants has been providing a prolific resource for healthcare aid in India (Mukherjee and Wahile, 2006). World Health Organization (WHO) has estimated that almost 80% of the world's population relies on traditional medicine for primary healthcare (Ekor et al., 2013). Given this huge reliance we need to evaluate the safety and efficacy of this traditional system of medicine before they are lost in this rapidly globalizing world (Mukherjee and Wahile, 2006).

The major tribal groups living in Purulia district are Santhalis, Oraons, Mundas, Bhumijs, Birhors, Gonds, Kharias, Mal Pahariyas and Hos. For primary healthcare, tribal communities rely mostly on age old folkloric medicine practiced by the traditional healers. Due to remoteness of villages

from conventional medical centres and inexpensiveness, efficacy, safety and availability issues, this age old traditional medicine has survived in the rigors of time and is still considered as the most popular therapeutic option among the ethnic groups residing in the rural parts of the district.However, very little work has been done to elucidate the phytochemistry (especially the marker compounds) and propagation aspects of indigenous medicinal plants. Since the underlying molecular mechanism of bioactivity of botanical preparations, crude extracts, fractions etc. mostly depend on its phytochemical content(s), scientific evaluation of phytochemical aspects of botanicals is absolutely necessary. Moreover, certain plants were noted by the authors to be used in a wide range of ethnomedicinal preparations and abundance of many of these plants is facing a decline in the last few years due to over exploitation, habitat destruction and lack of using alternative propagation strategies. However, little emphasize has been given on conservation aspects of rapidly declining medicinal plants' populations and on the correlation between phytochemistry and curative principles of medico-botanicals.Therefore, the present research program has been designed not only to explore the phytochemistry of the ethnobotanicals used by the aboriginals, but also to propagate and proliferate the widely used and/or conservationally challenged indigenous plants via micropropagation and other tissue culture techniques.Three plants *viz. Aristolochia indica, Bacopa monnieri* and *Rauvolfia sepentina*, very popularly used as ethnomedicine selected for further study for their *in vitro* propagation (to aid to their conservation as well as a source of *in vitro* germplasm *viz.* roots, leaves, whole plants, calli as sources of secondary metabolites with pharmacological significance) and phytochemical analyses to quantify important secondary metabolites (phytochemical markers) present in the three plants.

- *Aristolochia indica* was found to be one of the major herbal constituents in various monoherbal and polyherbal formulations used by the ethnic communities against various medical conditions without knowing the adverse side effects as a nephrotoxic (primarily contributed by its active ingredient Aristolochic acid. *Aristolochia* species cause aristolochic acid nephropathy (AAN), a renal interstitial fibrosis and upper urinary tract cancer (UUC) (Michl et al., 2013).

- *Bacopa monnieri* has been used as a popular memory enhancer in the district as well as other parts of India. The plant was ranked second in a priority list of the important Indian medicinal flora assessed considering medicinal properties, commercial exploitations and probable material for research and development (Anonymous 1997). However, bacosides, the active constituents reportedly responsible for the plants' neuroprotective and pro-memory activities are needed to be evaluated.

- *Rauvolfia serpentina* houses an array of indole alkaloids *viz.* reserpine, ajmaline, ajmalicine and many others. Since the plant is considered as a major tribal remedy

against many medical conditions, the phytochemical contents are needed to be evaluated.

2. Objective of the present study:

The present investigation aims at the following:

- Tribes residing in the villages of Purulia are affected by different types of medical conditions. Therefore, the first objective of the study was to elucidate the traditional medicinal knowledge of the botanicals used by various ethnic communities (Bhumijs, Birhor, Gond, Ho, Kharia, Mal Pahariya, Mundas, Oraon, Santhali etc.) of the district.
- This dataset was statistically analysed by calculating various quantitative indexes such as use value (UV), informants' consensus factor (FIC), fidelity level (FL) etc. Moreover, plants which were endangered or critically endangered in natural habitat due to habitat loss or over exploitation were also noted.
- Three plants *viz. Aristolochia indica, Bacopa monnieri* and *Rauvolfia sepentina* were selected for further study for their *in vitro* propagation (to aid to their conservation as well as a source of *in vitro* germplasms *viz.* roots, leaves, whole plants, calli as sources of secondary metabolites with pharmacological significance) and phytochemical analyses to quantify important secondary metabolites (phytochemical markers) present in the three plants.

3. Methodology

3.1. Ethnobotanical survey:

3.1.1 Study area and people

This westernmost district of West Bengal is located between 22°42'35" to 23°42'0" N latitude and 85°49'25” to 86°54'37” E longitude. Purulia is a part of the biogeographic zone 06B (Deccan Peninsula Chhotonagpur) with total forest coverage of 1857.26 km² which is 29.69 % of the total land. The total area of the district is 6259 km². The average temperature, relative humidity and rainfall range from 7°-45°C, 75-85% and 1240-1400 mm respectively. Undulated topography prevails in the area with an altitudinal variation of 250-650 m above mean sea level with a highest peak of 677m. The district has a total population of 2,927,965, more than 18% of which is tribal people. Almost 90% of the total population resides in the villages. This district is bordered on the east by two districts of West Bengal, Bankura and Paschim Medinipur, on the north by Bardhaman district of West Bengal and Dhanbad district of Jharkhand, on the west by Bokaro and Ranchi districts of Jharkhand and on the south by West Singhbhum and East Singhbhum districts of Jharkhand. The district is divided into 3 subdivisions *viz.* Purulia Sadar

East, Purulia Sadar West and Raghunathpur which are again subdivided into 20 blocks *viz.* Arsha, Baghmundi, Balarampur, Barabazar, Bundwan, Hura, Jhalda I and II, Joypur, Kashipur, Manbazar I and II, Neturia, Para, Puncha, Purulia I and II, Raghunathpur I and II and Santuri. The major ethnic groups residing in the district are Bhumijs, Birhor, Gond, Ho, Kharia, Mal Pahariya, Mundas, Oraon and Santhali. A huge number of the tribal people depend on the forest resources just for survival purpose. Forests play as the major source of food, fodder, timber and non-timber forest products to the inhabitants. Extreme weather, scanty and erratic rainfall and frequent draught, undulated and rolling topography, upland and monocropped cultivation, inadequate agriculture and lack of industrial development are responsible for its inclusion as one of the country's 250 most backward districts by the Ministry of Panchayati Raj, India in 2006.

2.2 Selection of informants

A total number of approximately 100 informants belonging to 9 tribal communities were chosen from different rural areas during the surveys. Most of the informants were either practicing traditional medicine for years derived from their ancestry or having knowledge of indigenous healing. Investigators have noted the informants' community, age, occupation (apart from practicing traditional healing), literacy and social acceptability of their healing ability. Most of the informants were found to be illiterate and school drop outs. Depending on the age, informants were classified into three groups *viz.* 20-35 years; 35-50 years and above 50 years.

2.3 Ethnobotanical Survey and data collection

Several ethnobotanical investigations were performed in different rural tribal villages of different blocks of the district to explore the indigenous use of medicinal plants. Previously prepared semi-structured questionnaire was used during the interview. An ethnobotanical data sheet was prepared which included scientific name of the plant, family, habit, vernacular or local name(s), plant part(s) used, method of preparation, route of administration, use of combining herbs and additives (if any), locality of collection, reporting tribe etc. along with the informant's name, age, sex, literacy, education, occupation etc. Informants were selected on the basis of their ethnomedicinal knowledge and ability to identify particular plants in the field by their vernacular names. Information regarding the plants' habit, habitat, flowering season, morphological features, presence/ absence of characteristic odour, toxicity etc. were noted and photographs of the plants in flowering/fruiting conditions were taken. Common plants were collected for identification and herbarium preparation. Less common, endangered and rare plants were not disturbed. The plants were initially identified from the flora books covering the district and the associated regions (Prain, 1903; Sanyal, 1994;) and verified by a taxonomist, Dr. Avinash Mundhra, Assistant Professor, RBC College, Naihati, West Bengal. The herbarium

specimens of the common plants available in flowering and/or fruiting condition were prepared in duplicate and assigned voucher number and preserved for future reference.

2.4. Data analysis

2.4.1. Informant consensus factor (F_{ic})

F_{ic} was calculated on the basis of the following equation (Heinrich et al., 1998); $F_{ic} = N_{ur} - N_t/(N_{ur}-1)$, where N_{ur} stands for the number of use reports for a particular use category and N_t stands for the number of taxa used for a particular ailment category by all informants. The homogeneity in the use of plants in the ailment categories among the informants of the study area is determined by the formula. As many species may be used to treat the same disease, this factor is used to determine the most used plant species to treat a particular ailment. A higher F_{ic} value is the indicative of using relatively few plants by the informants in the treatment of a particular ailment category whereas a lower F_{ic} value indicates the informants' disagreement of using a particular plant to treat a particular ailment category.

2.4.2. Fidelity level (FL)

The FL was calculated on the basis of the following formula (Friedmen et al., 1986); FL (%) = $(N_p/N \times 100)$ where N_p is the number of use-reports cited for a given species for a particular ailment category and N is the total number of use reports for any given species. FL is used to determine the most cited plant species to treat a particular disease by the informants of the study area. High FL of any given plant species indicates its widespread acceptance among the informants in the treatment of any given disease whereas low FL of a given plant indicates its use for many other purposes.

2.4.3. Use value (UV)

It was calculated on the basis of the following formula (Phillips et al., 1994). $UV = \sum U/n$ where U is the number of use reports for a given plant species cited by each informant and n is the total number of informants interviewed for a given plant. The relative importance of plant species used in the traditional medicine can be determined by UV where most frequently cited plant species show a higher UV and plants with lesser citation show a lower UV.

3.2. Plant Tissue Culture Experiment

3.2.1 Plant material and sterilization

Natural populations of plants were collected from Purulia district, Indi. Fresh young shoots were initially soaked in mild liquid detergent (Teepol®) for 15 min, followed by washing in running tap water for 20 min and rinsing in distilled water. The explants were surface sterilized

with 0.2 % Bavistin® (w/v) for 20 min and then treated with 0.1% (w/v) $HgCl_2$ for 10 min. Finally, the shoots were rinsed five times in sterile double distilled water.

3.2.2. Culture media and conditions:

MS (Murashige and Skoog, 1962) basal medium was used for the tissue culture experimentations. The medium was fortified with 3% (w/v) sucrose (Merck, India) and pH of medium was adjusted to 5.7 using 1 N NaOH before adding 0.8% (w/v) agar (Merck, India) in order to prepare semisolid medium. The medium (20 ml and 100 ml aliquots respectively) was poured into culture tubes and conical flasks (Borosil, India) and autoclaved at 121°C under 15lb/inch^2 pressure for 15 min. Various plant growth regulators (PGRs) such as auxins [IAA (indole-3-acetic acid), IBA (Indole-3-butyric acid), NAA (1-naphthaleneacetic acid), 2,4-D (2,4-dichlorophenoxyacetic acid)] and cytokinins [BAP (6-benzylaminopurine), kin (kinetin), TDZ (thidiazuron) (all PGRs were procured from Sigma-Aldrich, Germany)] and/or polyamines (Pas) [spermine/spm, put (Sigma-Aldrich, Germany), spermidine/spd (SRL, India), putrescine (put)] were used either singly and/or in various concentrations and combinations for shoot induction and elongation, multiplication, regeneration and *in vitro* rooting of explants. For shoot induction and multiplication, MS media supplemented with BAP, kin and TDZ along with NAA were used. For rooting *in vitro*, MS media fortified with different auxins were used whereas for callus formation and regeneration of shoots from organogenic callus, various auxins and cytokinins were added onto the MS media. All the cultures were incubated at 25 ± 2ºC in cool white fluorescence light and at 55 ± 5 % humidity with 16 h light/ 8 h dark photoperiod supplied by fluorescent tube light (Phillips, India), with a light intensity of 50 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD). Plantlets were cultured in supplemented MS medium *in vitro* and subcultures were performed at every 28-day interval. All experiments were repeated at least 3 times with 10 explants, with one explant per culture tube.

3.2.3 Induction and multiplication of multiple shoots:

About 2 cm of nodal explants were grown onto MS media supplemented with different concentrations and combinations of BAP, kin and TDZ, (0.5, 1, 1.5, 2 mg/l) for 4 weeks and the explants grown in the best multiplication medium was again subcultured in MS media containing spm, spd and put (0.5, 1 mM) with the best responsive concentration of cytokinin. As MS media supplemented with TDZ showed poor response when compared to BAP and kin supplemented media, TDZ was not used in combination with the PAs for the subsequent experimentations (depending on the plant species). Percentage of explants responding, the number and length of shoots produced by each of the responding explants were calculated after 4 weeks of incubation. MS basal medium without any hormone (MSO) was used as control. *In vitro* generated healthy shoots were maintained by regular subculturing onto MS media with same composition at 15 day intervals.

3.2.4. Regeneration of shoots from organogenic callus:

Nodal explants cultured in MS media supplemented with above mentioned concentrations and combinations of BAP, TDZ, kin (0.5, 1, 1.5, 2 mg/l), spm, spd and put (0.5, 1 mM) has produced green compact or white friable nodular base calli (NBC). After 4-6 weeks of incubation, multiplied shoots associated with NBC were excised and NBC were half inserted onto fresh MS medium fortified with similar combinations and concentrations of PGRs and PAs and subcultured every 4 weeks. Percentage of callus induction, average diameter of the NBC and number of regenerated shoots from NBC were recorded and compared with that of conventional multiplication in the similar media.

3.2.5. *In vitro* rooting and elongation:

Shoot tips (~2 cm in length) were aseptically excised from *in vitro* raised shoot clumps and transferred to the full and half strength MS medium supplemented with IBA and IAA individually or in combination with/without PAs *viz*. spm, spd and put for rooting and elongation of shoots. Average number and length of roots produced per shoot, average length of shoots and the number of nodes per elongated shoots were measured after 6 weeks of incubation.

3.2.6. Hardening and acclimatization:

Rooted plantlets were removed from the culture tubes/conical flasks, washed gently under running tap water to remove the agar and then placed in a 250 ml beaker containing tap water for 15 min. Thereafter the plantlets were transferred directly to pots containing autoclaved soil: sand: soilrite (2:1:2) and maintained in the culture environment for 4 weeks. To achieve high humidity, the pots were covered with polythene bags (10 cm × 8 cm) and irrigated daily with 1-2 ml tap water. After 4 weeks the plants were transferred to the larger pots with soil: soilrite (1:1) with no covering followed by transplantation into the field. The survival percentage of the hardened explants was recorded after 4 weeks of field transfer.

3.3. DNA isolation and clonal fidelity test:

In order to conduct genetic homogeneity test, *in vitro* regenerated plants were randomly selected along with the elite mother plant (MP). Young leaves from the plants were used for DNA extraction. The genomic DNA was extracted from the collected leaf samples following the Cetyl Trimethyl Ammonium Bromide (CTAB) protocol of Doyle and Doyle (1987) with slight modifications. DNA quality and quantity were determined through spectrophotometry at 260 and 280 nm respectively. The purity and integrity was later checked by performing 1.0% agarose gel electrophoresis and comparing the intensity of the resultant bands with 1kb DNA

ladder (Hi Media, India). 15 primers (6 RAPD and 9 ISSR primers) were selected from the 25 primers (10 RAPD and 15 ISSR primers) initially screened based on the production of clear, reproducible and scorable bands. Amplification for RAPD primers was carried out in a total volume of 25 µl containing 20 ng of genomic DNA, 2.5 µl of 10 X PCR buffer having 15mM $MgCl_2$, 0.02mM dNTPs, 1unit of Taq polymerase (Bangalore Genei, India) and 20ng RAPD primer (Eurofins Genomics, India). The RAPD amplification reactions were performed using the program of initial DNA denaturation at 94^oC for 4 mins, followed by 45 cycles of 1 minute denaturation at 94^oC, 1 min annealing at 32^oC and 1 min of extension at 72^oC with the final extension at 72^oC for 10 minutes. In case of ISSR primers, amplification was carried out in a total volume of 25µl containing 20ng of genomic DNA, 2.5µl of 10X PCR buffer containing 15mM $MgCl_2$, 0.2mM dNTPs, 1 unit Taq polymerase (Bangalore Genie, India) and 20ng of ISSR primers (Integrated DNA Technologies, India). The ISSR amplification cycles consisted of an initial DNA denaturation at 94^oC for 4 mins followed by 45 cycles of denaturation at 94^oC for 1 min, annealing at 45^oC for 1min and 2mins extension at 72^oC with a final extension at 72^oC for 10 mins.

The DNA amplification was performed in thermal cycler (Himedia, India) and the amplified fragments obtained were separated on 1.5% (w/v) agarose gel using 1 X TBE buffer. The separated fragments after electrophoresis were stained with ethidium bromide (0.5µg/ml) and finally visualized and photographed under Gel Documentation system (Syngene, UK). A 1kb DNA ladder (Hi Media, India) was employed to determine the size of unknown DNA fragments on the agarose gel. The amplification reactions of RAPD and ISSR primers were repeated twice to ensure the reproducibility of the banding pattern.

Data analysis: Clear, reproducible and well resolved bands were scored as present (1) or absent (0) for each RAPD and ISSR reaction. They were displayed as part of a binary data matrix and the intensity of band was not considered while scoring. The genetic similarity matrix between the mother plant (MP) and *in vitro* regenerated plants was calculated according to Nei and Li (1979). These similarity coefficients obtained were used to construct dendrogram through SYSTAT software using unweighted pair- group method with arithmetic mean (UPGMA) method.

3.4. Phytochemical Studies

3.4.1. Reserpine and Ajmalicine

The plant material of *Rauvolfia species* was collected. They were authenticated by the Department of Botany, Lovely Professional University, Phagwara (Punjab).

3.4.2. Preparation of Sample Solution

The air dried (25–30 °C) root parts of *Rauvolfia* species (1.0 g) were extracted three times with 20 mL of methanol for 45 min by reflux method in temperature controlled water bath by keeping the temperature 70 °C. The combined extracts were concentrated to dryness under vacuum at 45 °C. Dried extracts were re-dissolved in 1 mL of methanol and samples of varying concentration were spotted for quantification.

3.4.3. Preparation of Standard Solutions

Stock solutions of reserpine and ajmalicine (1 mg mL^{-1}) were prepared in methanol, and different amounts (2, 4, 6, 8, 10 and 12 µL) of these were loaded to a TLC plate, using Linomat applicator V for preparing six points calibration curves.

3.4.4. Chromatography

A Camag HPTLC system equipped with an automatic Linomat-5 automatic sample applicator, TLC scanner 3, and integrated software win CATS version version: 1.4.4.6337 was used for the analysis. HPTLC was performed on a pre-coated silica gel HPTLC $60F_{254}$ (10 cm × 10 cm) plate of 200 µm layer thickness for the quantification of reserpine and ajmalicine in *Rauvolfia species*. The samples and the standards were applied on the plate as 8 mm wide bands with constant application rate of 100 nL s^{-1}, an Linomat applicator V under a flow of N_2 gas, 15 mm from the bottom, 15 mm from the side, and the space between two spots was 14.4 mm of the plate.

3.4.5. Detection and Estimation of Reserpine and Ajmalicine

The linear ascending development was carried out in a Camag twin trough chamber (10.0 cm × 10.0 cm), which was pre-saturated with 25 mL mobile phase with Toluene: Ethylacetate: diethylamine (7:2:1) for 30 min, at room temperature (25 ± 2 °C) and 50 ± 5% relative humidity. The length of the chromatogram run was up to 80 mm. Subsequent to the development, the TLC plate was dried in a current of air, with an air dryer, in a wooden chamber with adequate ventilation. Quantitative evaluation of the plate was performed in the absorption-reflection mode at 268 nm, using a slit width 4.00 × 0.30 mm, data resolution 100 µm $step^{-1}$, scanning speed 20 mm s^{-1} and baseline correction was used. The source of radiation utilized was a Deuterium and tungsten lamp emitting a continuous visible spectrum 200 and 700 nm. Determination of reserpine and ajmalicine in different extracts was performed by the external standard method, using pure reserpine and ajmalicine as standards. Each analysis was carried out in triplicate.

3.4.6. Validation of HPTLC Densitometry Method

3.4.6.1. Linearity

Stock solutions of reserpine and ajmalicine were prepared in methanol and different amounts (2, 4, 6, 8, 10 and 12 µL) of these were loaded onto a TLC plate, using Linomat applicator V for preparing six points calibration curves. The regression equation and correlation coefficient were from calibration curves, for reserpine, $Y = 538 + 431.15\,X$ and 0.991 and for ajmalicine, $Y = -108.53 + 370\,X$ and 0.994.

3.4.6.2. Limits of Detection and Quantification

In order to estimate the limit of detection (LOD) and limit of quantification (LOQ), the blank methanol was spotted six times following the same method as explained. The signal-to-noise ratio was determined as 3:1 and 10:1 considered for LOD and LOQ, respectively. The limit of detection for reserpine and ajmalicine was 60 and 50 ng, respectively, and limits of quantification were 180 ng and 150ng respectively.

3.4.6.3. Accuracy

To the pre-analyzed sample, 50 and 100 µg each of reserpine and ajmalicine were added and the mixture was analyzed by the proposed method. The experiment was conducted in triplicate to check, recovery and accuracy of the system. The results are summarized in Table 1, showing the accuracy (expressed in recovery) of the method as the mean values of reserpine and ajmalicine.

3.4.6.4. Precision

System precision was performed by spotting six samples of same concentrations each from the stock solutions of reserpine and ajmalicine, (10 µg) and spotted on the silica gel $60F_{254}$ plate and analyzed with the proposed method. Six different samples of same concentrations were spotted on a plate and analyzed by the proposed method to determine variation arising from method itself.

3.4.2. Aristolochic Acid (After Agarwal and Ladhha, 2017)

3.4.2.1. Chemicals

N-Hexane, chloroform, acetone and methanol of laboratory grade were purchased. Standard aristolochic acid was purchased from Sigma.

3.4.2.2. Plant material

A. indica roots, stems, calli etc. was collected from natural populations as well as from *in vitro* raised samples.

3.4.2.3. Standard preparation for HPTLC analysis

Stock solution of aristolochic acid (1 mg/mL) was made with methanol. From the stock solution, 2 mL was taken to a 10-mL volumetric flask and the volume was made up with methanol (200 µg/mL).

3.4.2.4. Sample preparation

Five grams of each powdered drug was extracted separately with 50 mL methanol in Soxhlet for 5 hours. The extract was concentrated, transferred to a 25-mL volumetric flask, and the volume made up by methanol.

3.4.2.5. HPTLC analysis

Separation experiments were performed on silica-based HPTLC F254 plates (Merck, Darmstadt, Germany). Earlier, the silica plates were activated at 110°C for 15 minutes. Standard solution (1 mg/mL in methanol) was applied to acheive a five-point calibration curve corresponding to an amount of 0.4–2 µg/spot. All the runs were spotted in triplicate. A Camag HPTLC system equipped with an automatic Linomat-5 automatic sample applicator, TLC scanner 3, and integrated software win CATS version version: 1.4.4.6337 was used for the analysis. HPTLC was performed on a pre-coated silica gel HPTLC 60F254 (10 cm × 10 cm) plate of 200 µm layer thickness for the quantification. The samples and the standards were applied on the plate as 8 mm wide bands with constant application rate of 100 nL s−1, an Linomat applicator V under a flow of N2 gas, 15 mm from the bottom, 15 mm from the side, and the space between two spots was 14.4 mm of the plate.

3.4.3. Bacoside

3.4.3.1. Extraction and analysis of Bacosides

Extraction and HPLC analysis of bacoside A were carried out by following the method of Deepak et al. (2005). One gram of powdered shoot material was extracted in 100 ml of 70% methanol by heat refluxing and filtered through Whatman paper. The bacoside fractions were analyzed using HPLC system equipped with Phenomenex C_{18}, 5 µm (4.6 mm 9 250 mm) column, LC10AT VP lamps, SCL-10AVP system controller, SIL-10 AD VP auto injector, SPD-M10 AVP photodiode array detector. The mobile phase was a mixture of water containing 0.05 % (v/v)

orthophosphoric acid buffer and acetonitrile (70:30, v/v) at flow rate of 1.5 ml min^{-1} and column temperature was maintained at 30 ^{o}C. The detection wavelength was set at 205 nm. The bacoside A (1 mg/mL) were used as reference standards and an injection of (20 µL) was given to obtain the chromatograms of the standards. The dried extracts of *B. monnieri* in methanol from *in vitro* and *in vivo* cultured (2 mg/mL, injection volume 20 mL) samples were used to determine the quantity the constituents of bacoside A. The chromatography system was equilibrated by the mobile phase. Bacoside A standard was obtained from Sigma-Aldrich (USA).

4. Results

4.1. Ethnobotanical survey

4.1.1. Medicinal plants and traditional uses:

A total number of 300 plants belonging to various genera and families were reported to be used by the ethnic groups against different types of diseases. The plants are tabulated with their scientific name, family, habit, vernacular name, part used, used against, method of preparation and route of administration. The different habit types include trees, herbs, shrubs, climbers, vines. Percentage distribution of different plant parts used for the preparation of ethnomedicine includes whole plant followed by roots and fruits, stem, leaves, seeds and pod hairs. Use of above ground plant parts (considering whole plant in this category) was higher than that of below ground plant parts. Please refer to Tables 1-12 and Figs 1-19.

4.1.2. Method of preparation, route of administration and dosage:

Paste and hot water decoction were found to be the two most popular forms of medicine preparation. Different modes of preparation include paste, decoction, consumed raw, infusion, powder and juice. Paste, decoction, infusion and juice were usually prepared from the freshly harvested plant parts whereas the powder was kept in dry form for longer period of time for future use. Interestingly, the medicine prepared from the plant reproductive parts (flowers, fruits, seeds) were prepared during the particular flowering and/or fruiting season and stored to use throughout the year. Oral mode of administration was preferred over the topical mode. It was difficult to quantify the dosage since the traditional healers belonging to different tribal communities followed different dosages. Usually, 10-100 mg of freshly prepared plant material or powder was dissolved in 50-250 ml of water before consumption. The concentration and duration were found to be varied depending on the TH's suggestion. As a general rule, the dosage was found to be dependent on the patient's age, sex and general health. Children were prescribed a lesser dosage than the adults. Sugar, candy and honey were added to some

preparations especially for the children to reduce the bitter taste of herbal formulations. Decoction of *Aegle marmelos* was used with candy to make the preparation tastier. Powder of *Butea monosperma* and paste of *Phyllanthus emblica* were mixed with curdled milk and sugar to make the medicines more palatable. Three medicinal preparations were found to be used involving more than one medicinal plant. Root paste of *Argyreia speciosa* was prepared with the paste of *Asparagus racemosus* and *Hemidesmus indicus* roots. *Byttneria herbacea* root paste was made in combination with the fruit pulp of *Aegle marmelos* and the gum of *Anogeissus latifolia*. Leaf and stem paste of *Syzygium nervosum* was prepared with *Scoparia dulcis* plant paste. In general, the patients were advised to maintain a strict diet and withdraw from physical labour, alcohol and sex during the medication. Please refer to Tables 1-12 and Figs 1-19.

4.1.3. Informant's consensus factor, fidelity level and use value

The diseases or medical conditions were into several categories such as gastrointestinal disorders (GDs), sexual and gynecological disorders, urinary tract infections (UTI), neurological disorders, fever, snakebite etc. As for GDs, The Informant's consensus factor (Fic) was found to range between 0.57 and 1.00. The disease categories with highest use reports were diarrhea (with 47 use reports and 13 taxa) followed by amoebic dysentery (with 44 use reports and 13 taxa) and stomach pain (with 35 use reports and 12 taxa). In this present investigation, piles (Fic=1.00) had shown the highest Fic followed by bacillary dysentery (Fic=0.83), gastric ulcer, lack of appetite and intestinal worms (Fic=0.80). The lowest Fic was shown by cholera (Fic=0.57), followed by indigestion (Fic=0.64) and stomach pain (Fic=0.68). A high Fic indicates the use of relatively less number of taxa by a large number of informants whereas a low value is the indicative of informants' disagreement of using a particular plant in the treatment of a particular disease category. Highest Fidelity level (FL) (100%) was noticed for 8 plants used against amoebic dysentery. 100% fidelity level indicates a plant's popularity among the informants in the treatment of a particular disease. Please refer to Tables 1-12 and Figs 1-19.

4.2. Plant tissue culture

Nodal explants cultured on MS media supplemented with various concentrations of cytokinins exhibited axillary bud multiplication within 4 weeks. MS media supplemented with BAP and/or various polyamines were used depending on the plant species. For *Bacopa*, BAP (2.0 mg/l)+spd (1 mM) and in another set, kin (2.0 mg/l)+spd (1 mM) were found to be the best multiplication media producing 93.4 ± 0.00581 and 91.1 ± 0.00491 multiplied shoots per nodal explants respectively. Maximum average shoot length was achieved in MS media supplemented with BAP (2.0 mg/l)+put (1 mM) (average shoot length of 7.51 ± 0.00398 cm) and in another set, BAP (2.0 mg/l)+spd (0.5 mM) (average shoot length of 6.5 ± 0.00491 cm). Interestingly, almost

all concentrations and combinations of cytokonins (except TDZ) and PAs have showed enhanced rates of shoot multiplication. Full and half strength MS medium without auxins and also supplemented with IBA and IAA individually or in combination with PAs spm, spd and put produced slender roots with a number of laterals. After 6 weeks of culture, MS+ IBA (1.0 mg/l)+ spm (1.0 mM) produced highest average number of roots per shoot (33.3±0.0058) followed by MS+IBA (1.0 mg/l)+ spm (0.5 nM) producing 30.2±0.00406 roots/shoot. In all combinations, PAs combined with auxins have produced higher number of roots when compared to auxins alone. Highest root length (7.19±0.00254 cm) was achieved in MS medium supplemented with MS+IAA (1.0 mg/l)+ spm (1.0 mM). In all media compositions, the microshoots were found to elongate and after 6 weeks, maximum elongation (19.9±0.00254 cm) was found in MS+IAA (1.0 mg/l)+ spm (1.0 mM). The survival percentage of the hardened explants was found to be 100% in each experiment. For *A. indica*, the best rate of multiplication and regeneration from callus was observed in Schenk and Hilderbrand's (SH) media supplemented with BAP (2 mg/l). In *Rauvolfia*, MS media supplemented with BAP (2 mg/l) produced an average no. of 10 shoots per explant whereas the most prolific rooting was observed in MS basal media without any PGR.

4.3. Clonal Fidelity

In *Bacopa*, RAPD and ISSR markers were used to assess the clonal fidelity of in vitro raised plants and donor mother plant. Out of the 10 RAPD decamer primers initially screened, only 6 primers produced 16 reproducible and scorable bands with an average of 2.6 bands per primer. The sizes of amplified bands were in the range of 195-2500bp. The OPA-03 produced the maximum scorable bands of 7 while the least of only 1 band was generated by both OPD-08 and OPF-14 (Table 1). The RAPD profiles for the 5 selected primers showed monomorphic banding pattern indicating 100% genetic similarity between mother plant and in vitro clones (Figs. 1a,b &c). 15 ISSR primers were screened but only 9 primers produced 44 clear and reproducible bands with an average of 4.8 bands per primer and band size ranging from 300-2600bp. UBC-810 produced the highest number of 13 bands per primer while the minimum band of 1 was obtained for UBC-824 and UBC-830 (Table 1). 6 ISSR primers (UBC-820,UBC-822, UBC-823, UBC-828,UBC-830 and UBC-878) showed similar banding profiles between donor mother plant and in vitro progenies revealing 100% monomorphic characters (Figs. 2a & c), while the other 3 primers (UBC-810, UBC-824 and UBC-868) generated a total of 6 polymorphic bands exhibiting 15%, 100% and 42% degree of polymorphism respectively (Fig. 2c & Table 1). The pooled data analysis of 6 RAPD and 9 ISSR primers produced 60 distinct and scorable bands with an average of 4.0 bands per primer. The amplification products generated by each primer are in the range of 195-2600bp. The number of scorable bands for each primer varied from 01(OPD-08, OPF-14, UBC-824 and UBC-830) to 13 (UBC-810). Out of the 60 clear and reproducible bands recorded, 54 were found to be monomorphic displaying high degree of

monomorphism (90%) and lower polymorphic level (10%) between the mother plant and in vitro regenerants. The genetic identity established between the plants based on Nei and Lei's coefficient of similarity from RAPD and ISSR marker analysis was found to be high ranging from 0.88 (P1 and P4; P1 and P3; P1 and MP) to 1.00 (P3 and P4) (Table 2). P2 showed maximum genetic closeness to MP (0.97) while the least affinity to MP was exhibited by P1 (0.88). The dendrogram obtained from UPGMA analysis showed two distinct clusters. The P2, P3 and P4 which were closely identical to MP were grouped into the major cluster while the most genetically variant P1 was placed separately from the rest of the plants in another cluster (Fig. 3). In *Rauvolfia*, using ISSR Primers, UBC-810, UBC-820 and UBC-827 has produced 66.6% Polymorphisms.

4.4. Phytochemical analysis

Various compositions of mobile phases were tested to get better resolution of reserpine and ajmalicine. The resolution of reserpine and ajmalicine, with symmetrical and reproducible peaks, was achieved by using mobile phase consisting of toluene:ethyl acetate:formic acid (5:5:1). Peaks corresponding to reserpine and ajmalicine were recorded at R f 0.69 and 0.80, respectively. The methanolic extract of *Rauvolfia* species, when subjected to HPTLC, showed the presence of reserpine and ajmalicine peaks in all the samples. Comparison of the UV spectral characteristic of the peaks for standards of reserpine and ajmalicine, revealed the identity of reserpine and ajmalicine present in all samples. The calibration curves were linear in the range of 200, 400, 600, 800, 1000, 1,200 ng for reserpine and ajmalicine. Peak purity tests of reserpine and ajmalicine were also conducted by comparing UV visible spectra of reserpine and ajmalicine in *Rauvolfia* species standard and sample track. The higher contents (0.17% and 0.18% of ajmalicine and reserpine) in roots of *R. serpentina* and *R. tetraphylla* (0.16% and 0.15%) were recorded in roots of plant samples. In the present study we could observe the maximum contents of ajmalicine and reserpine in roots.

The HPLC of bacoside A showed four peaks at 8.520, 8.667, 9.373 and 9.633 min respectively. HPLC of bacoside A resulted into four peaks and they are assigned as (I) bacoside A3, (II) bacopaside II, (III) jujubogenin isomer of bacopasaponin C and (IV) bacopasaponin C. Linearity of detection at 205 nm for all four components of bacoside A was observed in the range of 4–20 mg (injected amount) with coefficient of determination (r2) values of>0.999 and>0.994. Relative standard deviation (RSD) values were<4.0 for all the concentrations injected (n = 3).

Please see Annexure for Tables

5. Concluding Remarks

- A thorough ethnobotanical survey was conducted in the Purulia district to study the vast yet less explored ethnobotanical knowledge of the tribal people. The dataset was evaluated with statistical methods.
- A rapid micropropagation and regeneration system was developed for the three plants. This served as a ready source of germplasms for medicinally important and/or endangered plant species and also for evaluating their bioactive phyto-constituents.
- Clonal fidelity of the in vitro raised germplasms was tested using RAPD based molecular markers.
- A detailed phytochemical profiling for the phytomarkers via High Performance Thin Layer Chromatography (HPTLC) and/or High Performance Liquid Chromatography (HPLC) to study phytochemical variations (population wise, altitudinal, seasonal, geographical, tissue specific, culture condition specific etc.) was carried out. Variations in some other secondary metabolites *viz.* Stigmasterol (antivenom), gallic acid and chlorogenic acid (antioxidants) were also compared in different plant species by HPTLC methods. Thus rapid and validated HPTLC and HPLC protocols established here may serve as useful protocols for phytochemical estimation, quality control and toxicity studies in various herbal preparations and drugs.

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